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Method of selecting targets for gene silencing
by RNA interference

Field of the invention

5 The invention relates to the preparation of
double-stranded RNA reagents for use in the specific
down-regulation of gene expression by RNA interference
by hybridisation to scanning arrays of antisense
oligonucleotides.

10

Background to the invention

RNA interference (RNAi) is a process of sequence-
specific gene silencing initiated by double-stranded
RNA (dsRNA) that is homologous in sequence to a region
15 of the silenced gene (Fire, A. Trends Genet. Vol. 15,
358-363, 1999; Sharp, P.A. Genes Dev. Vol. 15, 485-
490, 2001).

Elbashir et al. (Nature, 411, 494-498, 2001) have
20 demonstrated effective RNAi-mediated gene silencing in
mammalian cells using dsRNA fragments of 21
nucleotides in length (also termed small interfering
RNAs or siRNAs). These short siRNAs demonstrate
effective and specific gene silencing, whilst avoiding
25 the interferon-mediated non-specific reduction in gene
expression which has been observed with the use of
dsRNAs greater than 30bp in length (Stark G.R. et al.,
Ann Rev Biochem. 1998, 67: 227-264; Manche, L et al.,
Mol Cell Biol., 1992, 12: 5238-5248). Thus, siRNAs
30 represent promising candidate gene-specific
therapeutic agents, providing an alternative to anti-
sense oligonucleotides.

One potential problem to be overcome in the
35 development of siRNA agents for any given gene is the
selection of an appropriate region of the gene to

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target in order to achieve effective gene-silencing by RNAi.

5 In the field of anti-sense oligonucleotide technology suitable single-stranded antisense oligonucleotides for a given gene may be selected with the use of scanning arrays (Southern, E.M. et al., Nucleic Acids Res., 1994, 22(8): 1368-1373; Sohail, M. and Southern, E.M. "Using oligonucleotide scanning
10 arrays to find effective antisense reagents", *Methods in Molecular Biology*, vol. 170: *DNA Arrays: Methods and Protocols*, Ed J.B. Rampal, Humana Press Inc., Totowa, NJ; Sohail, M. et al., Nucleic Acids Res., 2001, 29(10): 2041-2051).

15 The present inventors have now shown that the sequences selected for use in single-stranded antisense oligonucleotides on basis of array screening can also be used as the basis of siRNA reagents that
20 are effective in gene silencing by RNAi.

Description of the invention

In accordance with a first aspect of the invention there is provided a method of preparing an
25 siRNA reagent for use in gene silencing of a target gene by RNA interference, which method comprises:

- (a) preparing a scanning array of antisense oligonucleotides spanning a region of a transcript of the target gene;
- 30 (b) hybridising to the array labelled transcripts of the target gene;
- (c) identifying an oligonucleotide within the array which hybridizes with the labelled transcripts; and
- 35 (d) preparing an siRNA reagent comprising a double-stranded RNA of identical sequence to the

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oligonucleotide identified in step (c).

As aforesaid, it is known to use scanning arrays of antisense oligonucleotides in order to identify regions of a particular transcript to target for inhibition of gene expression using antisense oligonucleotides. However, it was surprising to observe that sequences identified using the scanning array as being useful as antisense oligonucleotides may also mediate gene silencing by RNA interference because the mechanisms of action of antisense oligonucleotides and RNA interference are very different (see review by Brantl, S. Antisense-RNA regulation and RNA interference, Biochem Biophys Acta. Vol. 1575(1-3), 15-25, 2002).

The term "siRNA reagent" refers to a nucleic acid molecule that is capable of down-regulating expression of a target gene by RNA interference. The characteristics of siRNA reagents are generally known in the art. siRNA reagents generally comprise a region of double-stranded RNA, although as discussed below one or more bases in the double-stranded RNA may be replaced with DNA bases. The double-stranded RNA may be flanked by short single-stranded overhangs, as described below.

Scanning oligonucleotide arrays comprising oligonucleotides complementary to a target region of a mRNA transcript of the gene of interest may be synthesised using the methods which are known in the art and described, for example by Southern E.M. et al., Nucleic Acids Res., 1994, 22(8): 1368-1373 and Sohail, M. and Southern, E.M. "Using oligonucleotide scanning arrays to find effective antisense reagents", *Methods in Molecular Biology*, vol. 170: DNA Arrays:

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Methods and Protocols, Ed J.B. Rampil, Humana Press Inc., Totowa, NJ, the contents of which documents are incorporated herein by reference. A detailed protocol for synthesis of scanning arrays is also given in the accompanying examples.

Scanning arrays are a simple tool that allow combinatorial synthesis of a large number of oligonucleotides on a solid platform (typically glass or polypropylene, see note 1 in accompanying examples) in a spatially addressable fashion, and parallel measurement of the binding of all oligonucleotides complementary to the target mRNA.

The scanning arrays comprise sets of oligonucleotides of various lengths. A series of oligonucleotides, complementary to the target mRNA, is made by sequential coupling of nucleotides to a solid surface. The DNA synthesis reagents are applied to a confined area on the surface of the solid support using a mask (see below). The mask is shifted along the surface after each round of coupling, resulting in a series of oligonucleotides each complementary to a region of the target sequence.

The "gene of interest" may be essentially any gene for which it is desired to develop an siRNA/RNAi reagent. The method of the invention is of general utility, thus the precise nature of this gene (and the mRNAs transcribed therefrom) is not material to the invention. The "target region" may be a sub-fragment of the gene of interest which it is desired to test in order to identify region(s) which may potentially be useful targets for gene silencing by RNA interference. The "target region" may be an arbitrarily chosen sub-fragment of the gene of interest or may have been selected on the basis of an assay for potentially

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suitability as an RNAi target. For example, the "target region" may be one which is relatively accessible in the mRNA transcript because of a relative lack of secondary structure. Regions of mRNA transcripts which are potentially accessible for gene silencing may be identified by RNaseH mapping (see Sohail et al., Nucleic Acids Res., 2001, 29(10): 2041-2051).

10 The scanning arrays will generally contain all complements of the selected target sequence up to a maximum length determined by the size of the template and template displacement used in the synthesis of the scanning array (Southern et al. 1994, *ibid*).

15 Typically, the maximum length of the oligonucleotides in the array will be around 18-20 nt, but this may be varied if required. The short dsRNAs (siRNAs) used for RNA interference are typically 21-23 bp in length, hence it would be appropriate to include similar

20 length sequences in the array.

The scanning arrays are hybridised with a probe which is a transcript of the gene of interest labelled with a revealing label, which may be essentially any

25 type of revealing label which permits visualisation and quantitation of the hybridisation intensity. Radiolabels are particularly preferred. Suitable labelled RNA probes may be conveniently synthesised using standard techniques known in the art (see

30 accompanying examples).

Typically the hybridisation will be carried out at a temperature in the range 20-37°C. Hybridisation at 37°C is particularly preferred, since

35 oligonucleotides which hybridise at this temperature are more likely to be effective *in vivo*. The compositions of standard hybridisation buffers which

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are preferred for use with the arrays are given in the accompanying examples.

5 Oligonucleotides which hybridise to the array are identified and double-stranded RNA reagents (also referred to herein as RNAi reagents or siRNAs) having identical sequence are synthesised. The inventors have demonstrated by experiment that hybridisation to the scanning array is directly predictive of
10 effectiveness in RNA interference. In this context "identical sequence" is not intended to be interpreted literally as requiring 100% sequence identity. The sequence of the dsRNA may differ slightly from the antisense oligonucleotide (ASO) sequence. For example
15 the length of the dsRNA may be longer or shorter by several nt to optimise performance of the dsRNA. The RNA duplex will preferably be less than 30 bp in length, since duplexes of greater than 30 bp may induce non-specific interferon-mediated effects when
20 introduced into cells *in vivo*. RNA duplexes of 20-27 bp, and typically 20-24 bp, in length are particularly suitable as RNAi reagents. If the dsRNA is made longer than the ASO then the extra sequence may correspond to the "native" sequence of the mRNA. The
25 dsRNA may contain one or more substitute bases in order to optimise performance in RNAi. Substitution of even a single nucleotide may have a profound effect on activity of the RNAi duplex. The dsRNA may further contain non-natural bases or non-natural backbone
30 linkages, for example to enhance stability *in vivo* or enhance resistance to degradation by nucleases. The dsRNA may also include single-stranded overhangs at one or both ends of the duplex. In a particularly preferred embodiment the dsRNA may contain 3'
35 overhanging nucleotides, preferably 3' overhanging thymidines (dTdT) or uridines (UU).

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siRNA reagents may be formed of RNA/DNA chimeras. These chimeras include, for example, the siRNA reagents comprising a double-stranded RNA with 3' overhangs of DNA bases (e.g. dTdT), as discussed
5 above, and also siRNA reagents comprising a double-stranded "RNA" in which one or more of the RNA bases, or even an entire strand, are replaced with DNA bases.

10 In a further embodiment rather than being formed of two separate RNA strands annealed together, the siRNA reagent may comprise a dsRNA having a foldback stem-loop or hairpin structure, wherein the two strands of the dsRNA are covalently linked. RNAs having this structure are typical if the dsRNA is
15 synthesised by expression *in vivo* or by *in vitro* transcription. The precise nature and sequence of the "loop" linking the two RNA strands is generally not material to the invention, except that it should not impair the ability of the double-stranded part of the
20 molecule to mediate RNAi.

The double-stranded RNA will preferably comprise 20-27, or 20-24, consecutive nucleotides of the target mRNA sequence, since duplexes of this length are
25 particularly effective in RNAi.

Double-stranded RNAs may be synthesised *in vitro* using chemical or enzymatic RNA synthesis techniques well known in the art. In one approach the two
30 separate RNA strands may be synthesised separately and then annealed to form double-strands.

In a further embodiment, double-stranded RNAs may be synthesised by intracellular expression from a
35 suitable expression vector. Thus, the invention further provides a method of preparing an expression vector capable of expressing an siRNA reagent for use

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in gene silencing of a target gene by RNA interference, which method comprises:

- (a) preparing a scanning array of antisense oligonucleotides spanning a region of a transcript of the target gene;
- (b) hybridising to the array labelled transcripts of the target gene;
- (c) identifying an oligonucleotide within the array which hybridizes with the labelled transcripts;
- and
- (d) preparing an expression vector capable of expressing an siRNA reagent comprising a double-stranded RNA of identical sequence to the oligonucleotide identified in step (c).

A number of expression vector systems for *in vivo* expression of short double-stranded RNAs for use as RNAi reagents (also referred to as small interfering RNAs, or siRNAs) are known in the art. Generally, siRNAs are expressed as stem-loops, which may be rapidly processed within the cell to produce the "free" siRNA (see review by Tuschl, Nature Biotechnology, Vol. 20(5), 446-448, 2002). Vector systems for expression of siRNAs are often based on RNA Pol III promoters, since these are particularly suited to accurate expression of very short RNA sequences. Suitable vector systems are described in Brummelkamp, T.R. et al., Science, Vol. 296, 550-553, 2002; Lee, N.S. et al., Nature Biotechnology, Vol. 20, 500-505, 2002; Miyagashi, M & Taira, K. Nature Biotechnology, Vol. 20, 497-500, 2002; Paul, C.P. et al., Nature Biotechnology, Vol. 20, 505-508, 2002, the contents of which are incorporated herein by reference.

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In a further aspect the invention provides a

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method of preparing a pharmaceutical composition comprising an siRNA reagent capable of mediating gene silencing of a target gene by RNA interference, which method comprises:

- 5 preparing an siRNA reagent capable of mediating gene silencing of a target gene by RNA interference according to the method described above and formulating the siRNA reagent into a pharmaceutical composition comprising the siRNA reagent and one or
10 more diluents, excipients or carriers.

- siRNA reagents may be formulated into pharmaceutical compositions comprising a
15 therapeutically effective amount of the siRNA nucleic acid in combination with any standard physiologically and/or pharmaceutically acceptable carriers known in the art. "Pharmaceutically acceptable" means a non-toxic material which does not interfere with the activity of the pharmaceutically active ingredients in
20 the composition. "Physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, tissue or organism. Physiologically and pharmaceutically acceptable carriers may include diluents, fillers, salts,
25 buffers, stabilizers, solubilizers etc.

- For delivery into cells in vivo siRNAs may be formulated with lipid-based carriers including, for example, oil-in water emulsions, micelles, and
30 liposomes. Liposomes are the most preferred carriers, and there use is well known in the art. Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and OLIGOFECTAMINE™, which are formed of cationic lipids. Methods for making liposomes are
35 well known in the art and have been described in many publications. Liposomes may be targeted to a particular tissue by coupling the liposome to a

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particular tissue by coupling the liposome to a specific ligand, such as a monoclonal antibody, sugar, glycolipid or protein.

5 Liposomes may also be used to deliver vectors encoding siRNAs. In the field of human gene therapy it is well known to deliver expression vectors, such as plasmids, via nucleic acid-liposome complexes.

10 For use in human therapy, pharmaceutical compositions including the siRNAs of the invention will be administered to a patient in need of treatment in a "therapeutically acceptable amount". A
15 therapeutically acceptable amount is an amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response in the condition being treated. The precise amount of
20 the composition administered will, however, generally be determined by a medical practitioner, based on the circumstances pertaining to the disorder to be treated, such as the severity of the symptoms, the composition to be administered, the age, weight, and response of the individual patient and the chosen route of administration.

25 The invention will be further understood with reference to the following experimental protocols, together with the accompanying Figures, in which:

30 Figure 1 shows representative plots of hybridisation intensity across the array for (a) 15mers and (b) 18mers probed with labelled IGF1R mRNA.

35 Figure 2 illustrates the effect of ASOs and RNAi on IGF1R levels in MDA-231 human breast cancer cells. The cells were transfected at 30-40% confluence with phosphorothioate ASOs complexed with the lipid

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Cytofectin (Glen GSV). After 48hr the cells were lysed and equivalent amounts of soluble protein were separated by SDS-PAGE and immunoblotted for IGF1R and β -tubulin (loading control). The intensity of the autoradiographic bands was quantified by densitometry, and IGF1R levels were corrected for loading differences. The results are shown as % IGF1R level of that in cells transfected with the same concentration of an appropriate control. This was a scrambled control oligonucleotide for ASOs, and an inverted RNA duplex for RNAi.

Figure 3 illustrates the effect of ASOs and RNAi on IGF1R and IR levels in MDA-231 human breast cancer cells and ME melanoma cells. Cells were transfected using either Cytofectin (C) or Oligofectamine (O; Gibco BRL). After 48hr the cells were lysed and IGF1R and IR levels were determined by immunoblotting.

a) MDA-231 breast cancer cells transfected with ASO 2 (or scrambled control, Scr) or RNAi or inverted RNA duplex control (InvRNA) at 20 or 200nM.

b) MDA-231 cells were transfected with Oligofectamine and RNAi or Inverted control (InvRNA) at 0.1 - 10nM.

c) ME melanoma cells were transfected with Oligofectamine and RNAi or Inv control duplex at 5 - 500 nM.

Figure 4 illustrates the effects of various RNAi duplexes in MDA-231 breast cancer cells. MDA-231 cells were transfected with oligofectamine and 21mer RNA duplexes at 0.5, 5 and 50nm. After 48hr IGF1R expression was analysed by immunoblotting (panel (a)). Panel (b) is a graphical illustration, results are presented as % IGF1R level of that in cells transfected with the same concentration of an inverted control RNAi.

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- Figure 5 illustrates the activity of RNAi duplexes of 18, 21 or 24 nt in MDA-231 breast cancer cells. Panel (a) is an immunoblot showing the effect of various RNAis on IGF1R expression at 0.5 and 5nM; panel (b) is a graphical illustration showing the effect of RNAis of varying length, results are presented as % IGF1R level of that in cells transfected with the same concentration of an Inv2 control RNAi.
- Figure 6 illustrates the effect of RNAi duplexes on Akt phosphorylation in ME melanoma cells. ME cells were transfected with 100nM RNA duplexes. After 48hr the monolayers were disaggregated and half of each culture was treated with 10nM IGF-1 for 30 min. The cells were lysed and lysates were analysed by immunoblotting for IGF1R, phospho-Ser473-Akt and total Akt.
- Figure 7 illustrates growth in vivo in C57BL mice of B16 melanoma cells transfected with RNA duplexes. B16 melanoma cells were transfected with 200nM duplexes (RNA22=R2,22Inv=Inv2) or oligofectamine alone (OF) for three consecutive days. On the 4th day cells were injected into the flanks of C57BL mice, using 2.5×10^5 cells/mouse, using groups of 5 mice. Tumours were measured in 2 dimensions every 2-3 days. Tumour volumes were calculated as $n(a \times b^2)/6$ where a is the larger and b the smaller dimension.
- Figure 8 shows the complete cDNA sequence for human IGF1R. The region evaluated using the scanning array is underlined.

Example 1-Synthesis of a scanning array

In the following protocol references to Figures and Tables from "Sohail and Southern" refer to Sohail,

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M. and Southern, E.M. "Using oligonucleotide scanning arrays to find effective antisense reagents", *Methods in Molecular Biology*, vol. 170: DNA Arrays: Methods and Protocols, Ed J.B. Rampal, Humana Press Inc., Totowa, NJ.

Using a diamond-shaped or a circular reaction mask (Sohail and Southern, Fig. 1), it is possible to create arrays comprising sets of oligonucleotides of all lengths from monomers up to a maximum in a single series of couplings. The maximum length of oligonucleotides synthesised depends upon the ratio of the diagonal (for a diamond-shaped mask) or diameter (for a circular mask) of the mask to the displacement at each coupling step. For example, a diamond-shaped mask of 40 mm diagonal will produce 10-mers, 16-mers, or 20-mers using step sizes of 4 mm, 2.5 mm, or 2 mm, respectively. A diamond-shaped template creates a series of small diamond-shaped cells. The longest oligonucleotides are found along the centre line and the monomers are located at the edge (Sohail and Southern, Fig. 1). A circular template creates cells that differ in shape: along the centre line, they are lenticular, but off this line, they form a four-cornered "spearhead" that diminishes in size towards the edge. The arrays as synthesised are symmetrical above and below the centre line of the template and each oligonucleotide is represented twice allowing for duplicate hybridisation measurements.

For each length of oligonucleotides s , there are $N-s+1$ s -mers covering a total length of N bases. For example, if a 150 nt long sequence is covered in a 150 step synthesis, there will be 150 monomers and 131 20-mers. The last 20 positions in the sequence will be represented by shorter oligonucleotides only; in this

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case, from 19-mer to monomer. Therefore, for making 200 20-mers, an additional 19 nt synthesis steps need to be added at the end, i. e., total coupling steps = $N+1$.

5

Materials

Derivatisation of Glass

1. Glass cylinder and apparatus shown in Sohail and Southern, Fig. 3.
- 10 2. Glass sheets of required dimension (3 mm thick: Pilkington, UK).
3. 3-Glycidoxypentyl trimethoxysilane (98% v/v: Aldrich).
4. Di-isopropylethylamine (99.5 % v/v: Aldrich).
- 15 5. Xylene (AnalaR: Merck).
6. Hexaethylene glycol (97 % v/v: Aldrich).
7. Sulfuric acid (AnalaR: Merck).
8. Ethanol (AnalaR: Merck).
9. Ether (AnalaR: Merck).
- 20 10. Water bath at 80°C.

Making Reaction Masks

1. Stainless steel or aluminium square metal piece or PTFE (Teflon). Dimensions of the workpiece may vary
- 25 according to the size of the mask.
2. A centre lathe or a horizontal milling machine.
3. A drilling machine.
4. Abrasive paper from ~ P600 to P1200 (3M Inc., USA) and polishing grade crocus paper (J. G. Naylor & Co.
- 30 Ltd., Woodley, Stockport, Manchester, England).

Making Scanning Arrays

1. Solid support (derivatised glass or aminated polypropylene (Beckman Coulter, Inc., USA)).
- 35 2. DNA synthesiser (ABI).
3. A reaction mask of desired shape and size and assembly frame (see Sohail and Southern Fig. 4 for the

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assembly).

4. DNA synthesis reagents: standard dA, dG, dC and T phosphoramidites, oxidizing agent, acetonitrile, activator solution, deblock solution (all from Cruachem).

5. Reverse phosphoramidites bought from Glen Research

Deprotection of Arrays

1. Assembly for constructing deprotection bomb as shown in Sohail and Southern Fig. 5. The assembly consists of a high density polyethylene (HDPE) chamber, 4 mm thick silicon rubber gasket and a stainless steel plate of the dimensions of the HDPE chamber, and stainless steel M8 nuts and bolts.

2. 30% ammonia solution (AnalaR: Merck).

3. Water bath at 55°C.

In Vitro Transcription

1. Template DNA (at ~1 mg/mL).

2. T7 or SP6 RNA polymerase, transcription buffer (5X transcription buffer is, 200 mM Tris-HCl pH 7.9, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), recombinant RNasin®, 100 mM DTT and nuclease-free distilled water (Promega).

3. [α -³²P] UTP (3000 Ci/mmol) or [α -³³P] UTP (2500 Ci/mmol) (Amersham).

4. rNTPs (Pharmacia): ATP, GTP, CTP stored as 10 mM solution, and UTP as 250 mM solution in nuclease-free distilled water. Store all reagents at -20°C.

Quantitation of Transcripts

1. Scintillation counter (e. g., Beckman LS 1710).

2. Scintillation vials and scintillation fluid (Amersham).

Hybridisation, Imaging and Analysis

1. Hybridisation buffer (1M NaCl, 10 mM Tris-HCl pH

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- 7.4, 1 mM EDTA, 0.01 % SDS (w/v)) (see Note. 2).
2. 50-100 fmol radiolabelled transcript.
3. A glass plate of the size of the array when using
an array made on glass, a moist chamber (a large
plastic or glass lidded box containing wetted paper
towels) and an incubator set to the desired
temperature.
4. A hybridisation tube and oven used in standard
Southern hybridisation (e. g., Techne) when using an
array made on polypropylene.
5. Esco rubber tubing of OD 1 mm (Sterlin) for use in
Section 3.8.
6. Storage phosphor screen (Fuji or Kodak).
7. PhosphorImager or STORM (Molecular Dynamics).
8. A SUN Solaris work station for image analysis and
the computer software xvseq (L. Wand and J. K. Elder,
unpublished) (available by anonymous ftp at
<ftp://bioch.ox.ac.uk/pub/xvseq.tar.gz>).

Stripping of Arrays

1. Stripping solution (100 mM sodium
carbonate/bicarbonate buffer pH 10, 0.01 % SDS (w/v))
(see Note 3).
2. Geiger-Müller counter (Mini-Instruments Ltd.).

Methods

Derivatisation of Glass

1. Prepare a mixture of di-isopropylethylamine,
glycidoxypopyl trimethoxysilane and xylene (1: 17.8:
69, v/v/v) in a glass cylinder and completely immerse
the glass plates in the mixture. Incubate as shown in
Sohail and Southern Fig. 3 at 80°C for 9 h.
2. Remove the plates, allow them to cool to room
temperature, and wash with ethanol and then with ether
by squirting the liquid from a wash bottle.
3. Incubate the plates in hexaethylene glycol
containing a catalytic amount of sulfuric acid (~ 25

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mL/L) at 80°C for 10 h, with stirring.

4. Remove the plates, allow them to cool to room temperature, and wash with ethanol and ether. Air dry and store at -20°C.

5

Machining of Masks

1. Both stainless steel or aluminium can be used to make diamond-shaped and circular reaction masks. Circular masks are made using a centre lathe and diamond-shaped masks using a horizontal milling machine (see Note 4).
2. To make a diamond shaped mask from metal, hold the work piece at an angle of 45° to the axis of the bed of the milling machine (the diagonal of the diamond running parallel to the axis of the bed).
3. Machine the cavity to the required depth (generally between 0.5-0.75 mm) to create a reaction chamber. Machine the outer lands to a depth of approximately 0.5 mm to form the sealing edge (0.3-0.5 mm wide) (Sohail and Southern Fig. 4).
4. Using the smallest possible diameter cutter (~1.5 mm), radius the internal corners of the reaction chamber.
5. Finish the sealing edge by polishing flat with successively finer grades of wetted abrasive paper (from ~ P600 to P1200) and finally with a polishing grade crocus paper.
6. Drill holes of 1.08 mm diameter for reagent inlet and outlet, respectively, at the bottom and the top of the reaction chamber (in the corners of the diamonds). Inlet and outlet connections to the DNA synthesiser are made using standard 19SWG syringe needles (1.1 mm diameter) with chamfered ends ground off and de-burred (see Note 5).

35

Fabrication of Arrays

1. Cut glass or polypropylene to the correct size.

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- The process of making an array is the same when using either glass or polypropylene. Polypropylene has to be mounted on a glass plate, e. g., 3 mm thick soda glass (see Note 6). The total area covered by an array for N bases using a mask of diagonal or diameter D mm and step size l mm is $N \times l + D$ mm. 2-3 mm are added to margins to allow easy manipulations.
2. Fix the assembly (Sohail and Southern Fig. 4) to the front of a DNA synthesiser and connect its inlet and outlet to the synthesiser's reagent supply.
3. Program the DNA synthesiser with an appropriate synthesis cycle. A slightly modified cycle is used, for example, the one given in Table 1 (Sohail and Southern). Also check all the reagent bottles.
4. Enter the sequence (antisense strand) in 5' to 3' direction.
5. Mark the first footprint of the reaction mask on the support by placing it against the mask on the assembly in the desired starting position (see Sohail and Southern Fig. 6). A knife is used to make notches in polypropylene: A diamond scribe can be used to mark glass.
6. Tighten the plate against the mask with the pressure clamp to produce a seal (Sohail and Southern Fig. 4b). Sufficient pressure is applied to stop leakage (~ 500-800 Newton force) but not enough to create indentations in the polypropylene surface which can lead to leakage of reagent from the mask during subsequent synthesis steps.
7. Start the DNA synthesiser to go through the pre-programmed cycle to couple the appropriate nucleotide. The first condensation on the substrate is of base at the 3' end of the sequence.
8. After completion of the step during the interrupt (see Sohail and Southern Table 1 and Note 7), slacken the pressure clamp and move the plate one increment (Sohail and Southern Fig. 4b).

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9. Tighten the pressure clamp and start the synthesiser for the next nucleotide in the sequence. Continue the process until the full sequence length is synthesised (see Note 8).

5

Deprotection of Arrays

1. Place the glass or polypropylene array(s) into the HDPE chamber (see Note 9) and add 30% ammonia into the chamber to cover the array(s) completely.
- 10 2. Place the silicon rubber gasket around the rim of the chamber and the stainless steel plate on top of the gasket.
3. Place bolts through the metal plate, the gasket and the HDPE chamber, and tighten.
- 15 4. Incubate in a water bath at 55°C for 12-18 h in a fume hood.
5. Cool the assembly to 4°C before opening. The arrays are ready to be used in hybridisation at this stage.

20

Preparing and Quantifying Radiolabelled Transcripts

1. Set an *in vitro* transcription reaction (20 µL) by adding the following components to a microfuge tube at room temperature.
 - 25 5 X transcription buffer 4 µL
 - 100 mM DTT 2 µL
 - RNAasin™ 20 U
 - 10 mM ATP, GTP, and CTP 1 µL each
 - 250 mM UTP 1 µL
 - 30 Template DNA 2-3 µL (see Note 10)
 - [α-32P]UTP or [α-33P]UTP 2 µL
 - T7 or SP6 RNA polymerase 20 U
 - Total Volume 20 µL
 - Mix and incubate at 37°C for 1 h.
- 35 2. Remove 1 µL for quantitation (see below).
3. Remove unincorporated label by Sephadex® G25 column chromatography (see Note 11).

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4. Save 1 μL of the purified transcript for quantitation (see below).
5. Check the integrity of the transcript by denaturing polyacrylamide gel electrophoresis (10).
- 5 6. Add 10 μL of the scintillation fluid to the samples saved in Steps 2 and 4.
7. Mix by vortexing and count the samples in a scintillation counter for 1 min.
8. Calculate the per cent incorporation:
10 % incorporation = $\frac{\text{incorporated cpm} \times 100}{\text{total cpm}}$
9. Calculate the amount of RNA made:
Amount of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ = $\frac{20 \mu\text{Ci}}{3000 \mu\text{Ci/nmol}}$ = $6.6 \times 10^{-3} \text{ nmol}$
- 15 Amount of cold UTP = $1 \mu\text{L} \times 250 \mu\text{M} = 0.250 \text{ nmol}$
Total UTP = $6.6 \times 10^{-3} + 0.25 = 0.256 \text{ nmol}$
For a reaction with 50 % incorporation, the amount of UTP incorporated
= $0.256 \text{ nmol} \div 2 = 0.128 \text{ nmol}$
- 20 Supposing equal incorporation of all four nucleotides,
total nucleotides incorporated
= $0.128 \text{ nmol} \times 4 = 0.512 \text{ nmol}$
Amount of full-length transcript
= $0.512 \div \text{total length of transcript}$
- 25 Hybridisation to Arrays Made on Polypropylene
1. Place the array in the hybridisation tube, coiling it in a spiral.
2. Dilute the radiolabelled transcript in an
30 appropriate volume (10-20 mL depending upon the size of the array and the hybridisation tube) of hybridisation buffer. The mix should cover the array along the length of the tube.
3. Place items 1 and 2 in the oven at desired
35 temperature for 30 min. Also put approximately 100 mL of the hybridisation buffer in the oven: this is to be used to wash the array at the end of hybridisation.

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4. Pour the hybridisation mix into the tube containing the array and hybridise for 3-4 h.
 5. Remove the hybridisation mix. Briefly wash the array with the hybridisation buffer from Step 3, air dry, cover with cling film and expose to a storage phosphor screen for 16-20 h (see Note 12).
 6. Scan the screen on PhosphorImager or STORM and analyse the image using xvseq (see below).
-
- 10 Hybridisation to Arrays Made on Glass
 1. Clean the non-array glass plate with acetone and ethanol to ensure it is grease-free and siliconise it by treatment with dimethyl dichlorosilane solution and place it in lidded box. Also place moist paper towel in the box.
 2. Dilute the radiolabelled transcript in an appropriate volume of the hybridisation buffer (for example, for an array of 250 mm x 50 mm use 500-750 μ L).
 - 20 3. Place items 1 and 2 in an incubator at desired temperature for 30 min. Also put approximately 100 mL of the hybridisation buffer in the oven: this is to be used to wash the array at the end of hybridisation.
 4. Using a micropipette, pipette the hybridisation mix in a line evenly along the length of the non-array glass plate, avoiding formation of air bubbles.
 - 25 5. Starting at one end, carefully place the scanning array (face down) on top of the hybridisation mix. The mix will spread out and form a thin film between the two plates. Incubate for 3-4 h.
 - 30 6. Separate the plates from each other and wash the array plate with hybridisation buffer to remove unbound mix. Drain the plate, air dry, cover with cling film and expose to a storage phosphor screen for 35 16-20 h.
 7. Scan the screen on PhosphorImager or STORM and analyse the image using xvseq (see below).

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Alternative Hybridisation Protocol for Glass or Polypropylene

1. Assemble with clips the array plate (or polypropylene array pasted with PhotoMount™ on a glass plate) and the non-array plate, using rubber tubing as spacers on two sides.
2. Dilute the radiolabelled transcript in approximately 5-10 mL of hybridisation buffer.
3. Follow Step 3, Protocol 3.7.
4. Inject the hybridisation mix into the space between the two plates with a needle and syringe.
5. Incubate the assembly in horizontal position at desired temperature.
6. Follow Steps 6 and 7, Protocol 3.7.

Image Analysis

The hybridisation images are analysed using xvseq (see Sohail and Southern Fig. 7). This program reads and displays images generated by a PhosphorImager or STORM and can also perform standard image manipulation such as scaling, clipping and rotation. Although visual inspection of an image reveals the results generally, computer-aided analysis is needed to obtain quantitative information about hybridisation intensities and the oligonucleotide sequences that generated them. xvseq calculates and displays integrated intensities of the array oligonucleotides, each of which corresponds to an image cell formed by intersection of overlapping array templates.

The user can specify the template size, shape and location, step size between successive templates, as well as the sequence that was used to make the array. The template grid is superimposed on the image and the template parameters are adjusted interactively to achieve correct and accurate registration of the grid with the hybridisation pattern. It can be difficult to

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achieve precise registration by reference to the hybridisation pattern alone, especially, if the signals at either edge of the array are weak or undetectable. Avoid placing the template grid so that
5 it appears to be registered but is in fact misaligned by one or more template steps. Registration can be aided by the use of fixed reference points on an array such as those shown in Sohail and Southern Fig. 6.

10 Stripping of Arrays

1. The arrays can be used several times. To strip, heat an appropriate volume of the stripping solution to 90°C in a glass beaker.
2. Immerse the array in the hot stripping solution
15 and stir for 1-2 min.
3. Remove the array and monitor with a Geiger counter to confirm that most of the radiolabel has been removed. Repeat steps 1-2 if radioactivity on the surface of the array is still detectable.
- 20 4. Allow the array to cool down to room temperature and wash it thoroughly with nuclease-free distilled water, 70 % (v/v) ethanol and finally with absolute ethanol.
5. Air dry and store the array at -20°C until future
25 use.

Notes

1. The choice of array substrate material and attachment chemistry is important for making high
30 quality arrays. A flat, impermeable surface is required for *in situ* synthesis of arrays. Glass has a number of favourable qualities, including its wide availability, smooth surface, transparency, chemical stability and compatibility with the use of both
35 radiolabelled or fluorescence labelled nucleic acids targets. Glass is chemically derivatised as described in the methods section to produce a hexaethylene

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glycol linker which has a terminal -OH group that allows condensation of nucleotide phosphoramidites (Maskos, U., and Southern, E. M. (1992) Nucleic Acids Res. 20, 1679-1684.). Similarly, polypropylene also has favourable physical and chemical properties. Polypropylene is aminated to produce amine groups (Matson, R. S., Rampal, J. B., and Coassin, P. J. (1994) Anal. Biochem. 217, 306-310.) that also allow synthesis to oligonucleotides using standard CE nucleotide phosphoramidites.

For the array fabrication method described here, it is important that a tight seal is formed between the substrate material and the reaction mask. Metals form tight seal with polypropylene but not with glass. PTFE seals well against both glass and polypropylene.

2. 1M NaCl is used routinely. Alternative buffers are: (i) 1 M NaCl, 5-10 mM MgCl₂, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.01 % SDS (w/v), and (ii) 150 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.01 % SDS (w/v).

3. Addition of more than 0.01%SDS can damage arrays.

4. Circular masks can also be made from PTFE (Teflon). Diamond-shaped masks are more difficult to make with PTFE by the machining process but can be made by pressure moulding in a hydraulic press (~ 150 ton force) using a pre-machined die.

5. Holes should be made as close as reasonably possible to the sealing edge without damaging it. Care must be taken to de-burr fully the holes at the point of entry into the reaction chamber. For PTFE masks the holes should be 1.0 mm diameter which make virtually 100% leak-tight seal. In the case of metal masks, the 0.02 mm interface indicated above also provides a

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leak-tight seal without the use of any additional sealer. Care must be taken not to insert the end of the syringe needle into the reaction chamber void.

5 6. Unlike glass, polypropylene is not rigid and thus
needs to be mounted on a solid, flat surface for its
precise movement against the reaction mask during
synthesis. Even mounting of polypropylene on glass is
important to produce a good seal between the sealing
10 edge of the reaction mask and the polypropylene
surface. Glass used must be clean and free from dust
particles because they can cause bulging of the
polypropylene which can hinder the formation of a
proper seal. A very thin layer of PhotoMount™ (3M
15 Inc, USA) which can be used to paste polypropylene to
glass, should be sprayed on glass and not
polypropylene.

20 7. At the start of each synthesis cycle, an
interrupt step can be introduced to halt the process
at the first step of the next nucleotide condensation
cycle to allow the operator to move the plate and
restart the program. Alternatively, a long wait step
at the beginning of the program can be introduced (see
25 Sohail and Southern Table 1) if the operator does not
wish to use the interrupt step. The operator is also
advised to consult the user's manual for the DNA
synthesiser.

30 8. With the use of standard phosphoramidites in the
synthesis, the oligonucleotides are attached to the
solid support at their 3' ends. Reverse
phosphoramidites can be used to make oligonucleotides
that are attached at their 5' ends.

35

Iodine is used as an oxidising agent. At lower
temperatures it will take longer to reach the top of

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the reaction cell. Iodine can also be replaced with sulfurising agent (Cruachem) to make arrays of phosphorothioate oligonucleotides.

- 5 9. When using the standard phosphoramidites, the exocyclic amines of the bases are protected chemically to prevent side reactions during synthesis. These protecting groups need to be removed from the coupled bases before hybridisation.

10

Before deprotection, detach the polypropylene arrays from glass by peeling from one end. PhotoMount can be removed with ethanol, acetone or dichloromethane.

15

10. An internally radiolabelled RNA is used as target to hybridise to a scanning array which is generated by in vitro transcription, carried out in the presence of [α - 32 P]UTP or [α - 33 P]UTP (or [α - 32 P]CTP) using an appropriate DNA template. A plasmid containing the desired DNA fragment under the transcriptional control of a T7 or SP6 promoter (such as pGEM: Promega) can be used as template. The plasmid is linearised with an appropriate restriction endonuclease to produce transcripts of defined length without contaminating vector sequence. Alternatively, a template with T7 or SP6 RNA promoter can also be generated using the polymerase chain reaction: primers are used to amplify the required fragment from a plasmid, genomic DNA or cDNA, such that the sense primer has a T7 or SP6 promoter leader sequence (Sohail and Southern Table 2) added at the 5' end.

25

30

11. Sephadex® G25 columns are available from several commercial suppliers including Promega and Pharmacia. Spin columns made in-house, as described in (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular

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Cloning: A Laboratory Manual. Cold Spring Harbor, NY.), can also be used.

12. For hybridisation below 37°C, care must be taken
5 not to touch the plates because this can lead to melting of short duplexes. For hybridisation below room temperature, the cling film and the phosphor screen must be cooled to hybridisation temperature and exposed at the same temperature.

10

Example 2-Use of scanning array to select RNAi reagents

- The following is give by way of example of the utility of scanning arrays in the selection of RNAi
15 reagents. The invention is of general utility in the selection of RNAi reagents, thus it is not in any way intended to limit the scope of the invention to this specific example.

- 20 Scanning arrays complementary to the region of the IGF1R mRNA from position 537-685 were prepared using the standard techniques described above. The maximum length of the oligonucleotides in the array was either 18 or 20 nt.

25

- In this study hybridisation to the arrays was carried out at physiological temperature (37°C) in addition to room temperature (23°C), in order to select sequences which are more likely to have
30 activity in intact cells. The arrays were probed with labelled IGF1R mRNA, and also with labelled insulin receptor mRNA in order to identify oligonucleotides which have high specificity for IGF1R mRNA.

- 35 IGF1R template/probe preparation

Human IGF1R cDNA in plasmid pCVN was a generous gift from Renato Baserga. The 5' region of IGF1R cDNA

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was cloned into vector pBluescript KS- (Stratagene) using restriction sites HindIII (pCVN-derived site at 5' end of IGF1R cDNA) and Asp718 (cuts IGF1R cDNA at position 1581). This construct (template 1, 1.6kb) included approximately 100bp of polylinker sequence between the T7 promoter and the start of the IGF1R sequence. It was thought that this extraneous vector-derived sequence might influence folding of the transcript. Therefore this region was shortened by restriction digest using NotI, filling in with Klenow enzyme, digestion with EcoRV and re-ligating to make construct 2 (1.6kbΔ55). Both constructs were linearised at the 3' end of the IGF1R insert using Asp718, to make templates for in vitro transcription. End-labelled transcripts 1 (1.6kb) and 2 (1.6kbΔ55) (see Figure 1) were generated using T7 RNA polymerase and γ -³²P-GTP (Amersham Pharmacia).

Results

Representative plots of hybridisation intensity across the array for 15mers and 18mers probed with labelled IGF1R mRNA are shown in Figure 1. The plots are annotated to show the position of selected individual oligonucleotide sequences. In each case the sequence given corresponds to the sense strand of the IGF1R mRNA.

Relative hybridisation intensities for selected oligonucleotides were calculated relative to the most strongly hybridising sequence.

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Table 1: Relative hybridisation intensity

Oligo	Sequence	Hyb intensity	Rel hyb intensity
ASO2	ggctcttcacacatcgg	56,026	1.00
ASO4	gcggtagttgtactcattgt	>33,162	>0.59
ASO1	ggcttctcctccatgggc	15583	0.28
ASO3	attgttgatgggtggtcttct	>7048	>0.13
ASO5	gtgggtccagcagcggtagtt	>4150	>0.07
ASO6	cttattccccacaatgta	560	0.01

Example 2-Synthesis of RNAi duplexes

Double-stranded RNA duplexes for RNAi corresponding in sequence to certain of the ASOs identified on the basis of the array screening as hybridising strongly or weakly with IGF1R mRNA were synthesised, together with corresponding inverted controls, mouse homologs and mutant variants (see Table 2). RNA oligonucleotides were synthesised and HPLC purified at Cruachem, Glasgow. Lyophilised oligoribonucleotides were reconstituted in nuclease-free water and diluted to 50µM. Complementary strands were annealed in 100mM potassium acetate, 30mM Hepes-KOH pH 7.4, 2mM magnesium acetate, as described (Elbashir et al., Nature, 2001, www.dharmacon.com) to give a final concentration of 20µM duplex. Duplex formation was checked by electrophoresis through 5% low-melting point agarose (NuSieve GTG, BioWhittaker Molecular Applications) in 1xTBE.

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Table 2: RNAi duplex sequences

5	R2	GCCGAUGUGUGAGAAGACCTT TTCGGCUACACACUCUUCUGG	SEQ ID NO:23
	R2 (18mer)	GCCGAUGUGUGAGAAGTT TTCGGCUACACACUCUUC	SEQ ID NO:24
	R2 (24mer)	GCCGAUGUGUGAGAAGACCACCTT TTCGGCUACACACUCUUCUGGUGG	SEQ ID NO:25
10	R2 (27mer)	GCCGAUGUGUGAGAAGACCACCAUCTT TTCGGCUACACACUCUUCUGGUGGUAG	SEQ ID NO:26
	Inv2	CCAGAAGAGUGUGUAGCCGTT TTGGUCUUCUCACACAUCGGC	SEQ ID NO:27
	Mut2	GCCGAUGUGUGUGAAGACCTT TTCGGCUACACACACUUCUGG	SEQ ID NO:28
15	MouseR2	GCCCAUGUGUGAGAAGACCTT TTCGGGUACACACUCUUCUGG	SEQ ID NO:29
	R6	CUACAUUGUGGGGAUAAGTT TTGAUGUAAACACCCCUUAUUC	SEQ ID NO:30
	Inv6	GAAUAAGGGGUGUACAUCTT TTCUUAUCCCCACAAUGUAG	SEQ ID NO:31
	MouseR6	CUACAUUGUGGGGAACAAGTT TTGAUGUAAACACCCCUUGUUC	SEQ ID NO:32

For comparison purposes the following antisense
oligonucleotides were also synthesised:

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Table 3: Antisense oligonucleotides

	ASO1 (18mer)	GGCTTCTCCTCCATGGTC	SEQ ID NO:1
	ASO1 (20mer)	CGGCTTCTCCTCCATGGTCC	SEQ ID NO:2
5	Mouse ASO1	GGGCTTCTCCTCCAATGTCC	SEQ ID NO:3
	Scr1	TCTTCCGCGACTTGCTCCGC	SEQ ID NO:4
	Mouse Scr1	CTGTTACCCGTGCACCCTGT	SEQ ID NO:5
	ASO2 (18mer)	GGTCTTCTCACACATCGG	SEQ ID NO:6
	ASO2 (20mer)	TGGTCTTCTCACACATCGGC	SEQ ID NO:7
10	Mouse ASO2	TGGTCTTCTCACACATGGGC	SEQ ID NO:8
	Scr2	CCTGTACGCGTTGATCTCCA	SEQ ID NO:9
	Mouse Scr2	AGTCGCCTAGTCGAGTCCTT	SEQ ID NO:10
	ASO3	ATTGTTGATGGTGGTCTTCT	SEQ ID NO:11
	Scr3	TCATGCTTGTTGATGTGGTT	SEQ ID NO:12
15	ASO4	GCGGTAGTTGTACTCATTGT	SEQ ID NO:13
	Scr4	TGTCGTTGAGTTGATCGTA	SEQ ID NO:14
	ASO5	GTGGTCCAGCAGCGGTAGTT	SEQ ID NO:15
	Scr5	GACGTTAGCGTGCGATGTGC	SEQ ID NO:16
	ASO6	CTTATTCCCCACAATGTA	SEQ ID NO:17
20	Mouse ASO6	CTTGTTCCTCCCCACAATGTA	SEQ ID NO:18
	Scr6	CATAGCACATTCTCTCA	SEQ ID NO:19
	Mouse Scr6	ACCGTTCATCAGTTCCT	SEQ ID NO:20
	TSS	TCCTCCGGAGCCAGACTT	SEQ ID NO:21
25	ScrTSS	CAGCTACTCGCATGCTGC	SEQ ID NO:22

Example 3-Testing of RNAi duplexesCell culture and transfection

30 The human and murine cell lines used in this study were cultured in RPMI 1640 plus 10% FCS at 37°C in a humidified atmosphere of 5% CO₂. The cultures

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were negative when tested for *Mycoplasma* infection. Cultures were passaged the day before transfection to achieve 30-50% confluence the following day. Initial transfections of ASOs used 1-2.5 $\mu\text{g}/\text{ml}$ Cytofectin (Glen Research, Sterling, VA), according to the manufacturer's instructions. Latterly Oligofectamine (Life Sciences) has been used for all ASO and RNAi transfections. The method was as described (see manufacturer's instructions and www.dharmacon.com) with minor modifications. For transfection of monolayers seeded in 6 well plates, 5 μl phosphorothioate oligonucleotide or RNA duplex at 100x final concentration were mixed with 250 μl serum-free Optimem (Gibco-BRL). In a separate tube 2 μl Oligofectamine was mixed with 68 μl Optimem. After 10min incubation at room temperature the contents of the two tubes were mixed and incubated for a further 25min at room temperature. Monolayers were washed with 1-2ml Optimem. To the cells were added 175 μl Optimem followed by the 325 μl complexes. Volumes were scaled up by factors of 2.22 or 6.05 for transfection in 60mm or 100mm dishes respectively. Cultures were incubated at 37°C for 4hr, and then 50 μl FCS and 3.5ml RPMI plus 5% FCS were added to each well. After 48hr incubation effects on IGF1R and IR expression were determined by immunoblotting. Some transfected cultures were disaggregated using 3mM EDTA in PBS for 2-4min, washed in serum-free RPMI, divided into two aliquots and treated with 10nM Long R3-IGF-I (GroPep, Adelaide) or diluent for 30min at 37°C prior to lysis.

Immunoblotting

IGF1R expression was assessed by immunoblotting as previously described (Macaulay et al 2001). After washing in ice-cold PBS, cells were lysed in 50mM Hepes pH 7.5, 100mM NaCl, 10mM EDTA, 1% Triton-X-100, 4mM sodium pyrophosphate, 2mM sodium orthovanadate,

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10mM sodium fluoride, 1mM PMSF, 2 μ g/ml each leupeptin and aprotinin. Lysates were centrifuged for 15minutes at 14,000g, the protein concentration of supernatants was measured using BCA assay reagent (Pierce), and equivalent amounts of protein were separated on 7.5% SDS-PAGE gels and transferred to nitrocellulose. Target protein levels were assessed using antibodies to the β -subunit of the IGF1R or IR (Santa Cruz), phospho-Ser 473 Akt or total Akt (Cell Signalling, New England Biolabs) or β -tubulin (Sigma). Primary antibodies were detected with HRP-conjugated secondary antibodies (Dako), and ECL Plus (Amersham Pharmacia).

15 Results

(1) Down-regulation of IGF1R in MDA-231 cells by antisense oligonucleotides and RNAi:-comparison of the activity of ASOs and RNAi.

20 ASOs and RNAi duplexes were transfected into MDA-231 cells using Cytofectin or Oligofectamine, as described above. After 48hr the cells were lysed, and IGF1R and IR levels were measured by immunoblotting. The intensity of the autoradiographic bands was quantified by densitometry, and IGF1R (or IR) levels were corrected for loading differences. The specific IGF1R or IR results are presented as % IGF1R (or IR) level of that in cells transfected with the same concentration of an appropriate control. This was a scrambled control oligonucleotide for ASOs, and an inverted RNA duplex for RNAi. The results are shown in Table 4 and Figure 2.

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Table 4: Effect of IGF1R-ASOs on IGF1R and IR expression in MDA-231 cells

ASO	Protein Level (% control)			
	IGF1R		IR	
	30nM	300nM	30nM	300nM
1	66±23	38±6	112	135
2	84±11	47±5	nd	nd
3	105±18	104±22	nd	nd
4	73±2	49±2	97	92
6	nd	100	nd	100
TSS	141±10	64±19	144	59

- 15 The effects of an ASO and RNAi duplex of equivalent sequence (ASO2 and R2) on IGF1R and IR expression were compared in MDA-231 human breast cancer cells and ME melanoma cells. The transfection protocol was as above, using either Cytofectin (C) or
- 20 Oligofectamine (O; Gibco BRL). After 48hr the cells were lysed and IGF1R and IR levels were determined by immunoblotting. Representative immunoblots are shown in Figure 3.
- a) MDA-231 breast cancer cells transfected with ASO 2 (or scrambled control, Scr) or RNAi (sequence R2 given above) or inverted RNA duplex control (InvRNA) at 20 or 200nM.
- 25 b) MDA-231 cells were transfected with Oligofectamine and RNAi or Inverted control (InvRNA) at 0.1 - 10nM.
- 30 c) ME melanoma cells were transfected with Oligofectamine and RNAi or Inv control duplex at 5 - 500 nM.
- Quantitative analysis of these results is incorporated
- 35 into the graph of ASO effects in MDA-231 (Fig 2).

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It is clear that RNAi causes much more profound inhibition of IGF1R expression than occurs with ASOs. Note that the quantification methods may not be linear at very low protein levels, and it is possible that these analyses underestimate the true extent of IGF1R downregulation. For example the immunoblots (Figure 5) show almost complete inhibition of IGF1R expression (even on these overnight exposures) in MDA-231 breast cancer and ME melanoma cells treated with 10-200nM RNAi.

A second RNAi (R6 in Fig 4; sequence R6 in Table 2) was synthesised corresponding to ASO 6, which hybridizes to IGF1R mRNA on the array with a relative intensity of 0.01. While this does cause downregulation of the IGF1R, the effects were significantly less potent than that of the RNAi corresponding to ASO2 (R2 in Fig 4; sequence R2 in Table 2). RNAi R2 is more effective than RNAi R6 in the following cell lines:

DU145	prostate
A549	NSCLC
UC101	ovary
25 U20S	osteosarcoma
MCF7	breast (ER positive)

This result indicates that intensity of hybridisation to the scanning oligonucleotide array is predictive of the activity of RNAi reagents.

The inhibitory effect of RNAi R2 is partially, though not completely, blocked by the presence of a single base pair mutation (sequence Mut2, in Fig 4), see Figure 4, compare R2 with Mut2. The effect of R6 was less than the effect of the mutant duplex Mut2. This indicates that the efficacy of synthetic 21mer

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RNAi molecules is influenced by secondary structure in, and hence access to, the target region of the mRNA.

- 5 (iv) Comparison of RNAi duplexes R2 and R6 in a range of human and murine cell lines

10 Tumour cells were transfected with 10nM duplexes and IGF1R levels were measured after 48hr. After correction for loading differences IGF1R levels in cells transfected with RNAi were expressed as % of levels in cells transfected with equivalent inverted control duplex.

- 15 Table 4: Effect of R2 and R6 RNAi duplexes in human and murine cell lines

Cell line	Cell type	R2	R6
DU145	Human prostate cancer	34±13	74±8
20 A549	Human non-small cell lung	22±2	102±16
ME	Human melanoma	53±3	104±5
B16	Murine melanoma	45±4	72±14

- 25 In some cell lines eg human ME melanoma and A549 NSCLC, 10nM R6 was essentially inactive. In all cases R2 caused more profound IGF1R downregulation than R6. This suggests that the structural features dictating access are robust and conserved between different cell lines and species.
- 30

(v) Effect of RNAi duplexes of varying length

- 35 Compared with the effects of R2, IGF1R downregulation is less profound after treatment with

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an 18mer duplex (sequence as R2 but lacking the 3 RNA bases at the 3' end). In contrast to the results of Elbashir et al. (2001), a 24mer R2 duplex was observed to be as effective as R2 at 100nM. Results are illustrated in Figure 5. Panel (a) is an immunoblot showing the effect of various RNAis on IGF1R expression at 0.5 and 5nM; panel (b) is a graphical illustration showing the effect of RNAis of varying length, results are presented as % IGF1R level of that in cells transfected with the same concentration of an Inv2 control RNAi. A 27mer R2 duplex was also observed to be as effective as the R2 20mer and 24mer duplexes (data not shown).

15

Example 4-Differential effects of RNAi duplexes on IGF signalling

ME cells were transfected with 100nM RNA duplexes. After 48hr the monolayers were disaggregated and half of each culture was treated with 10nM IGF-1 for 30 min. The cells were lysed and lysates were analysed by immunoblotting for IGF1R, phospho-Ser473-Akt and total Akt (see Figure 6).

25

The IGF1R downregulation that follows transfection with R2 was sufficient to inhibit IGF-I-induced Akt phosphorylation, while R6 caused modest IGF1R downregulation that was not sufficient to block IGF-I signalling to Akt (Figure 6). This is significant because it indicates that R2, which corresponds to an ASO hybridising strongly to the scanning array, not only caused more potent IGF1R downregulation but that this translated to a significant biological advantage; Akt phosphorylation is the major anti-apoptosis pathway downstream of the IGF1R.

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Example 5-Growth of cells transfected with RNAi
duplexes in vivo

5 B16 cells were transfected with 200nM RNA
 duplexes (RNA22=R2,22Inv=Inv2) or oligofectamine alone
 (OF) for three consecutive days. On the 4th day cells
 were injected into the flanks of C57BL mice, using
 2.5×10^5 cells/mouse, using groups of 5 mice. Tumours
10 were measured in 2 dimensions every 2-3 days. Tumour
 volumes were calculated as $\pi(a \times b^2)/6$ where a is the
 larger and b the smaller dimension.

 B16 cells transfected with R2 showed reduced
15 growth rate in vivo, (syngeneic C57BL mice) compared
 with cells transfected with inverted control RNA
 duplex (Figure 7). This results indicates that an
 RNAi reagent selected on the basis of hybridisation to
 a scanning array is effective in vivo.

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Claims:

1. A method of preparing an siRNA reagent for use in gene silencing of a target gene by RNA interference, which method comprises:
- 5 (a) preparing a scanning array of antisense oligonucleotides spanning a region of a transcript of the target gene;
- 10 (b) hybridising to the array labelled transcripts of the target gene;
- (c) identifying an oligonucleotide within the array which hybridizes with the labelled transcripts; and
- 15 (d) preparing an siRNA reagent comprising a double-stranded RNA of identical sequence to the oligonucleotide identified in step (c).
2. A method of preparing a pharmaceutical composition comprising an siRNA reagent capable of mediating gene silencing of a target gene by RNA interference, which method comprises:
- 20 preparing an siRNA reagent capable of mediating gene silencing of a target gene by RNA interference according to the method of claim 1 and formulating the siRNA reagent into a pharmaceutical composition
- 25 comprising the siRNA reagent and one or more diluents, excipients or carriers.
3. A method of preparing an expression vector capable of expressing an siRNA reagent for use in gene silencing of a target gene by RNA interference, which method comprises:
- 30 (a) preparing a scanning array of antisense oligonucleotides spanning a region of a transcript of the target gene;
- 35 (b) hybridising to the array labelled transcripts of the target gene;

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(c) identifying an oligonucleotide within the array which hybridizes with the labelled transcripts; and

5 (d) preparing an expression vector capable of expressing an siRNA reagent comprising a double-stranded RNA of identical sequence to the oligonucleotide identified in step (c).

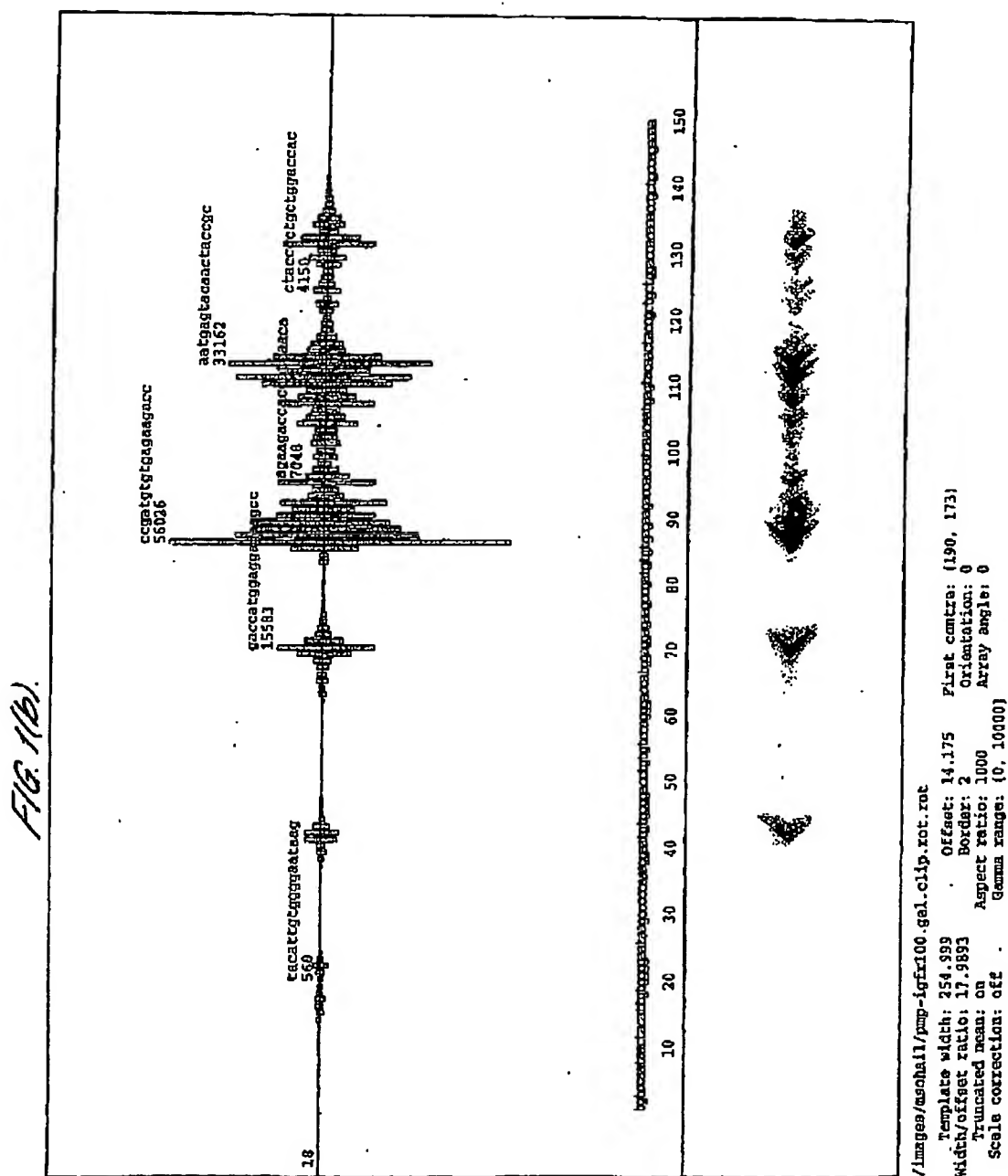
10 4. A method of preparing a pharmaceutical composition comprising an expression vector capable of expressing an siRNA reagent for use in gene silencing of target gene by RNA interference, which method comprises:

15 preparing an expression vector capable of expressing an siRNA reagent for use in gene silencing of a target gene by RNA interference according to the method of claim 3 and formulating the expression vector into a pharmaceutical composition comprising the expression vector and one or more diluents,
20 excipients or carriers.

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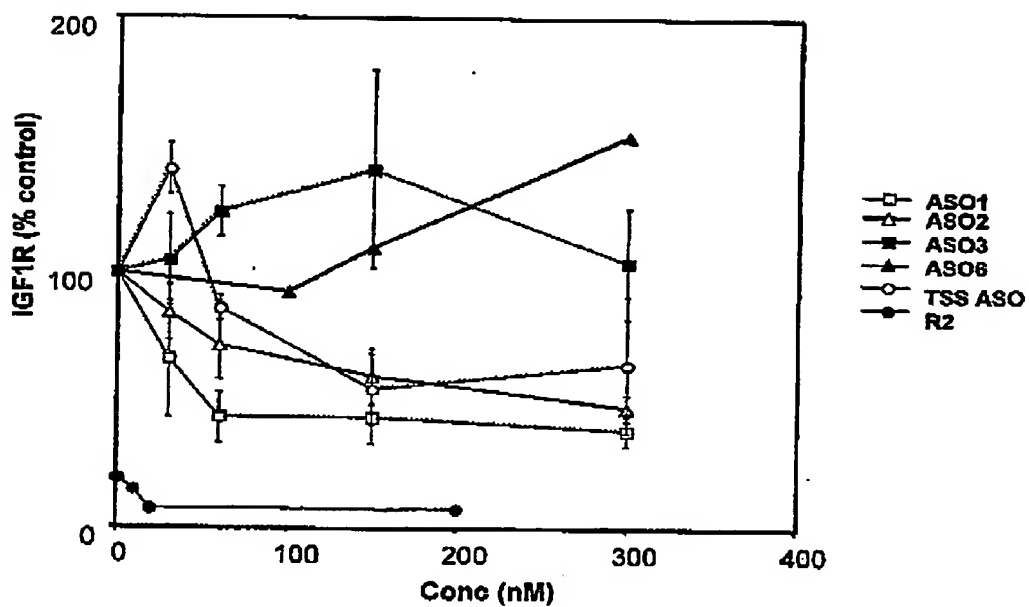


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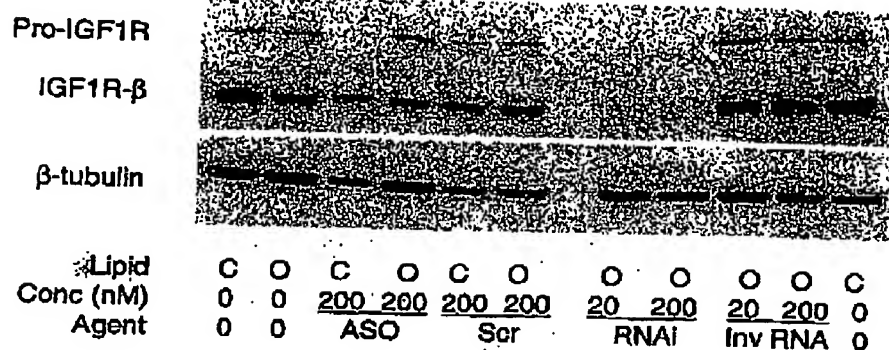
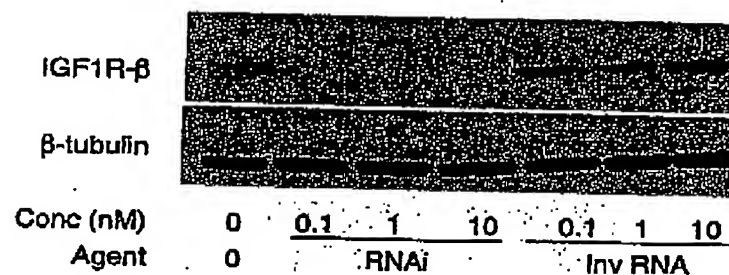
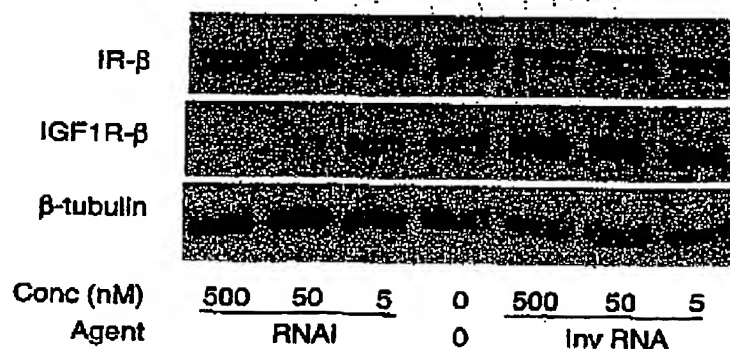
FIG. 2.



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*FIG. 3.***a****b****c**

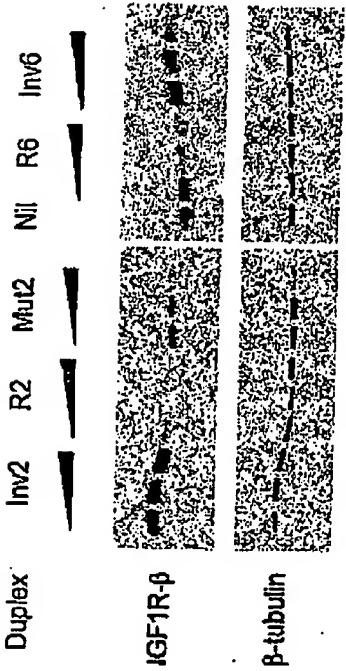
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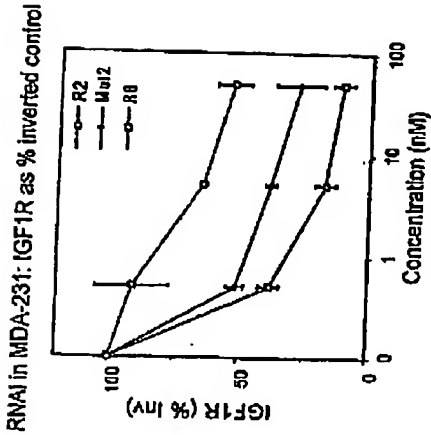
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FIG. 4.

(A)



(B)



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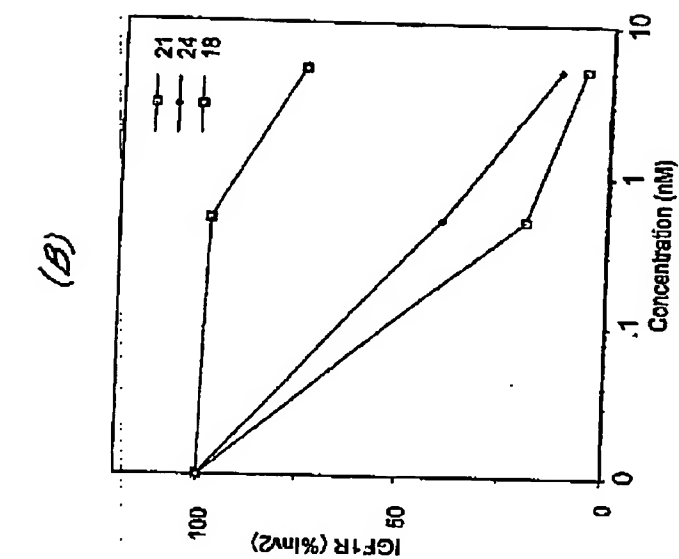
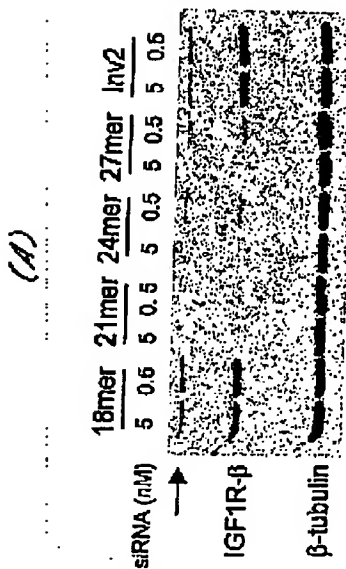


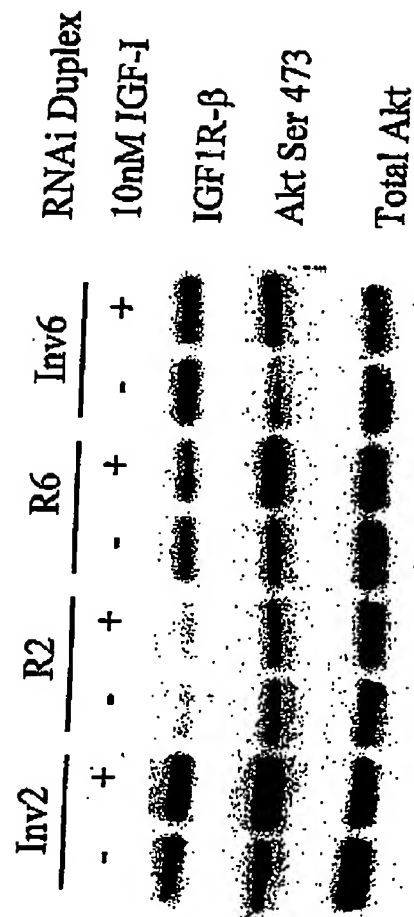
FIG. 5.



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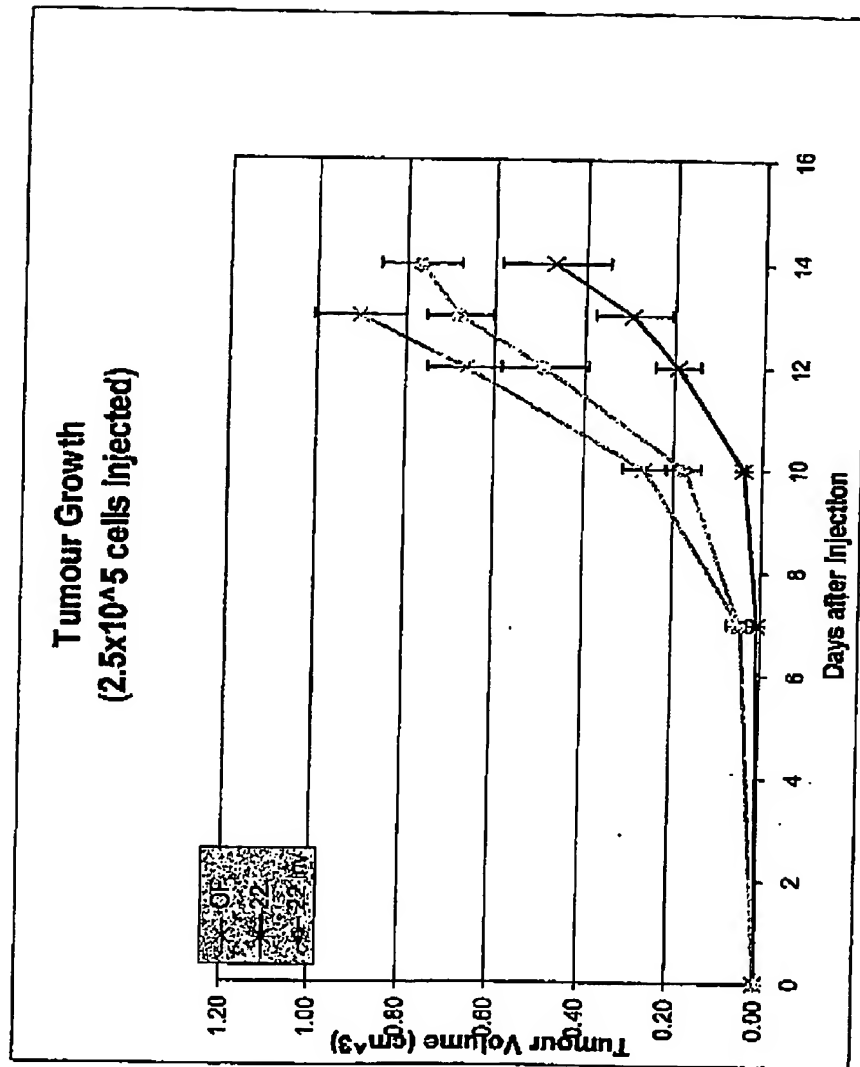
FIG. 6.

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FIG. 7



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*FIG. 8.***IGF1R cDNA sequence bases 1-1000.**

```
1      TTTT TTTT TTTT TTTGAGAAAGGGAATTT CATCCCAAATAAAAGGAATGAA
51     GTCTGGCTCCGGAGGAGGGTCCCCGACCTCGCTGTGGGGGCTCCTGTTTC
101    TCTCCGCCGCGCTCTCGCTCTGGCCGACGAGTGGAGAAATCTGCGGGCCA
151    GGCA TCGACATCCGCAACGACTATCAGCAGCTGAAGCGCCTGGAGAACTG
201    CACGGTGATCGAGGGCTACCTCCACATCCTGCTCATCTCCAAGGCCGAGG
251    ACTACCGCAGCTACCGCTTCCCCAAGCTCACGGTCATTACCGAGTACTTG
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351    CCTCACGGTCATCCGCGGCTGGAAACTCTTCTACAACTACGCCCTGGTCA
401    TCTTCGAGATGACCAATCTCAAGGATATTGGGCTTTACAACCTGAGGACA
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Underlined are translation start site and region screened by array.

Latter covers 150nt representing bases 536-685 of human IGF1R sequence.

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